NITROGEN ACQUISITION BY WHEAT, CANOLA, AND SOYBEAN INOCULATED WITH THE N_2-FIXING BACTERIUM *GLUCONACETOBACTER DIAZOTROPHICUS*

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ABSTRACT

Demands for food and fiber continue to increase, as does the global population. As a consequence, the use of nitrogen (N) fertilizers also is increasing globally. However, as the use of N fertilizer increases, so do concerns about the impacts of N loss to the environment. Efforts to reduce reliance on synthetic N fertilizers involve multiple strategies, including the development of biofertilizers. Capitalizing on microorganisms that fix N is one potential avenue for reducing the need for fertilizer N. There exist in nature many species of soil microorganisms (collectively referred to as diazotrophs) that can fix atmospheric N. One group of diazotrophs, including the species *Gluconacetobacter diazotrophicus*, are endophytes that invade plant roots and either colonize the spaces between the cells (intercellular) or the cell matrix itself (intracellular). Azotic North America recently began marketing a *G. diazotrophicus*-based inoculant (Envita™) reported to have beneficial effects including N fixation and yield improvement in crops as diverse as rice, wheat, corn, and soybean. This research aimed to quantify N-fixation in crops important to Saskatchewan producers (e.g., wheat, canola, and soybean) that were inoculated with Envita™. Experiments relied on the use of ^{15}N tracing to verify N-fixation and a new droplet digital polymerase chain reaction (ddPCR) method to verify the presence of *G. diazotrophicus* in plant tissues of the Envita™-inoculated crops. Different methods of introducing the inoculant to the plants also were evaluated. On average, soybean showed no significant difference between treatments, though a few plants did show evidence of N-fixation. Moreover, while some treatment differences in ^{15}N content were found for wheat and canola when the inoculant was introduced using the root bath application (RBA) method, the inoculation status from the ddPCR was inconsistent and yielded conflicting results. The data suggest that Envita™ has the potential to provide some N through N fixation to wheat, canola, and soybean but that more research is needed to optimize inoculation approaches and to fully understand the conditions necessary for N fixation to take place.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBCH	Biologische Bundesanstalt, Bundessortenamt and Chemical industry
BNF	Biological nitrogen fixation
DAG	Days after germination
Di-H_2O	Deionized water
FAO	Food and Agriculture Organization of the United Nations
Gd	*Gluconacetobacter diazotrophicus*
HSD	Honest significant difference
N	Nitrogen
NA	North America
NRC	National Research Council
PM	Particulate-matter
V	Vegetative growth stage
WHO	World Health Organization

1. INTRODUCTION

Nitrogen (N) is one of the most important nutrient elements and is a basic component of plant and animal life—indeed, N is one of the building blocks of amino acids, proteins, and DNA. Although N is abundant in the atmosphere, comprising 78% by volume, it is not readily available for use by most organisms. Nitrogen availability is crucial for the agriculture industry, but there are limited pathways for the conversion of gaseous N into available N. In nature, gaseous N is converted by lightning into ammonia (NH_3) and nitrate (NO_3^-), which then enter the soil through precipitation. Additionally, some bacteria, such as N-fixing bacteria, can convert atmospheric N_2 into available N. However, with the human population expanding dramatically, the amount of nitrogen and other nutrients needed to grow food has also increased. To meet these ever-increasing demands, synthetic N fertilizers have become a critical component of modern agriculture.

The history of fertilizer use can be traced back thousands of years. Ancient farmers used manure and ash to improve the productivity of their land (Andersen, 2018). While the use of organic fertilizers, such as manure, has a long history, the history of inorganic fertilizers is relatively short. In 1906, Fritz Haber invented the Haber-Bosch process, which initiated the era of mass production of synthetic N fertilizers, which now dominate the fertilizer market. In 2019, the Food and Agriculture Organization of the United Nations (FAO) reported that the supply of N fertilizer was 161 million tonnes, while the demand for N fertilizer was 107 million tonnes (FAO, 2019). Additionally, as the price of agriculture products reached record highs in 2021, the FAO forecast that the demand and supply of fertilizers will continue to increase in the coming years.

While synthetic fertilizers are the backbone of modern agriculture, the side effects of their use, particularly the use of synthetic N fertilizer, have gained notice and sparked concerns. The amounts of NO_3^- entering aquatic ecosystems (including groundwater) and nitrous oxide (N_2O, a potent greenhouse gas) entering the atmosphere are of particular concern (Byrnes, 1990). For example, the World Health Organization (WHO) has determined that groundwater containing more than 50 mg NO_3^- L^{-1} (WHO, 2017) is unsuitable for drinking, and the maximum acceptable concentration of nitrate in Canada is 45 mg L^{-1} (Health Canada, 2006). Likewise, emissions of

fertilizer-derived N_2O from agricultural soils account for 72% of total anthropogenic N_2O emissions in Canada (Rochette et al., 2008). Nitrogen fertilizers are particularly problematic because the N can enter the environment, and produce negative environmental impacts, along multiple pathways. Depending on the soil type and surrounding environment, N fertilizer can be lost due to leaching, denitrification, nitrification, volatilization, and immobilization (Farrell et al., 2006).

Some bacteria can convert atmospheric N_2 into a usable form of N via the process known as biological nitrogen fixation (BNF). These bacteria, known as diazotrophs, possess the nitrogenase enzyme that allows them to convert atmospheric N_2 into ammonia (NH_3). Diazotrophs can be free-living (without direct interaction with plants), associative (living in the rhizosphere or, in the case of endophytic diazotrophs, inside the plant), or symbiotic (in a mutualistic relationship with the plant). The most well-known of these microorganisms are members of the genus Rhizobium, which form close associations with legumes that under optimal conditions convert atmospheric N_2 into NH_3 for the plant while acquiring other essential nutrients from the plant (Wagner, 2011). Rhizobia are a diverse group of N-fixing soil bacteria that can contribute up to 80% of a plants N needs (Purwaningsih et al., 2021). A review by Herridge et al. (2008) found that globally pulses (grain legumes) can fix as much as 2.95 million tonnes of N while oilseed legumes can fix as much as 18.5 million tonnes. Soybean (*Glycine max*) is the dominant oilseed legume and in the Herridge et al. (2008) study accounted for 16.4 million tonnes of fixed N, or 77% of the total N fixed via legume production.

While rhizobia live in the soil and can be reintroduced to legumes when growing conditions are favourable, the rhizobia-legume association is highly specific; i.e., a given rhizobial strain can form a symbiotic relationship with only a limited number of host plants (Sharma et al., 1993). Therefore, efforts to extend the benefits of BNF to non-legume crops such as wheat and canola have focused on N-fixing bacteria that lack host specificity—namely the associative diazotrophs (Santi et al., 2013; Pankievicz et al., 2019). One such N_2-fixing bacterium, *Gluconacetobacter diazotrophicus*, was discovered in sugarcane in Brazil in 1988 (Cavalcante & Dobereiner, 1988). *G. diazotrophicus* is a non-nodulating endophytic N-fixing bacterium that can survive in low pH, high sucrose, and high salt environments (Cavalcante & Dobereiner, 1988; Chawla et al., 2014). Additionally, *G. diazotrophicus* has been found in other plants such as rice and corn (Cocking et al., 2006). Thus, it was believed that the bacterium had the potential to be inoculated into other

plants such as soybean, wheat, and canola. Indeed, in 2006 Professor Edward Cocking from the University of Nottingham published a study describing the inoculation of *G. diazotrophicus* into maize, rice, wheat, oilseed rape, tomato, and white clover—confirming that *G. diazotrophicus* could be inoculated into a broad range of important crop species (Cocking et al., 2006). Eventually, Azotic Technologies (Nottingham, UK) took on the challenge of developing the *G. diazotrophicus* into a commercial inoculant product and in 2014 announced the development of key N-fix technology that had successfully improved rice production and could replace as much as 50% of fertilizer N (AzNA, 2022). This N-fix technology was later commercialized as Envita™ in the UK and registered for use in the US and Canada in 2019.

Although used primarily in corn production, Azotic North America (AzNA) is looking to expand its range of crops to include wheat and canola, which are the two dominant crops produced in Saskatchewan. For example, in 2022 Saskatchewan accounted for 53% (i.e., 9.5 MT) of total canola production in Canada and 43.8% (i.e., 14.8 Mt) of total Canadian wheat production. Therefore, the potential impact of Envita™ to provide economic and environmental benefits (e.g., a reduction in N fertilizer usage and its attendant reduction in N_2O emissions) in Saskatchewan is huge. Moreover, although Saskatchewan does not have the climate for broad-scale soybean production, changing climate patterns and the increasing price of soybean products has generated considerable interest in growing soybean—especially in southeastern Saskatchewan. At present, however, short-season soybean varieties adapted to Saskatchewan conditions have lower protein contents than their long-season counterparts grown in Manitoba and the central US (Brar & Lawley, 2020; Canadian Grain Commission, 2021). Thus, producers in Saskatchewan are looking for ways to increase soybean protein content, which could potentially benefit from endophytic N_2 fixation provided Envita™ can be successfully inoculated into soybean.

This project was developed to determine (i) if and how *G. diazotrophicus*—the active ingredient of Envita™—can be inoculated into wheat, canola, and soybean, and (ii) whether the inoculated crops can fix atmospheric N through an associative relationship with *G. diazotrophicus*. My hypothesis was that the Envita™ inoculant would result in wheat, canola, and soybean plants containing measurable amounts of *G. diazotrophicus* and that this would result in the plants accumulating significant amounts of biologically fixed N from the atmosphere.

The thesis is organized in a traditional style with an Introduction (Chapter 1) followed by a Review of the Literature (Chapter 2), Methods and Materials used in the study (Chapter 3), a

presentation of the results of the research (Chapter 4), followed by a Discussion of the Results (Chapter 5), and the Conclusions (Chapter 6), which summarizes the key findings and takeaways of this research and identifies possible future research opportunities. A listing of the references cited throughout the thesis is provided in Chapter 7, which is followed by an Appendix containing a detailed description of the method used to detect the presence of *G. diazotrophicus* in the inoculated plants.

2. LITERATURE REVIEW

2.1. The Importance of Nitrogen

Nitrogen (N) is one of the most important and abundant elements on earth, with dinitrogen (N_2) comprising about 78% of the earth's atmosphere. Nitrogen is also one of the basic building blocks of life—being required to produce amino acids, proteins, nucleic acids (DNA and RNA), adenosine triphosphate (ATP), and (in plants) chlorophyll (Postgate, 1998; Bernhard, 2010). However, though abundant in the atmosphere, N_2 is inert and unreactive and, consequently, is not directly available to plants. In general, plants access N from the soil through their root system, and though recent evidence suggests that plants can acquire significant amounts of N in organic form (Farzadfar et al., 2021), inorganic forms of N in the soil—primarily ammonium (NH_4^+) and nitrate (NO_3^-)—are considered the major sources of plant available N. Over the short- and long-term, however, crop removal can dramatically reduce the supply of available N in the soil, resulting in the need for N fertilizers to remain productive.

Humans have understood the importance of providing nutrients to crops since ancient times, with records of fertilizer use to promote crop growth dating back thousands of years. Indeed, it is believed that manure was used as fertilizer as far back as 8000 years ago (Balter, 2013). On the other hand, the history of synthetic N fertilizer usage is relatively short—tracking back primarily to the development of the Haber-Bosch process in the early 1900s (Russel & Williams, 1977; Prasad & Shivay, 2021). Nitrogen being one of the most important crop nutrients, the global supply and demand for N fertilizers has increased steadily since 1960 (FAOSTAT, 2023). For example, the Food and Agriculture Organization (FAO) of the United Nations reported that total N fertilizer use in agriculture increased more than 10-fold between 1961 and 2020; i.e., from 11 Mt to 113 Mt (FAOSTAT, 2023). And in 2021, China, India, and the United Sates accounted for approximately 40% of the total agricultural N fertilizer used (FAOSTAT, 2023).

Wheat is one of the most important food crops in the world and requires a significant amount of N to maintain the production needed to meet the ever-increasing demands of a growing global population. Although grain yield and protein content in wheat are positively related to N

supply, excessive N can result in a reduction of yield (Fig 2.1). Likewise, although protein content can benefit from the addition of N, there is a trade-off between protein content and grain yield at high N levels (Selles & Zentner, 2001; Ma et al., 2019; Jaenisch et al., 2020).

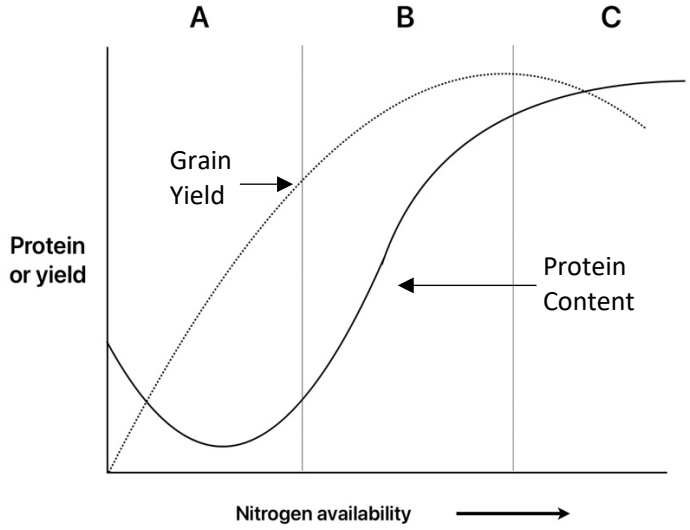

Fig. 2.1. Ideal yield and protein response to nitrogen availability in wheat crops (adapted from Selles & Zentner 2001). Region **A** represents a small amount of nitrogen fertilizer; region **B** represents the optimal amount of nitrogen fertilizer, and region **C** represents an overuse of nitrogen fertilizer.

Canola is primarily used for oil production and studies have shown that while an increase in N fertilizer can increase the yield of canola seeds, the oil content of the seeds tends to decrease at high soil N levels (Taylor et al., 1991; Yahbi et al., 2022). Soybean is also a major source of oil, as well as of protein and carbohydrates, and though most soybean production relies on biological nitrogen fixation (BNF), soybean yield and protein content can be increased by the application of N fertilizer (Morshed et al., 2008). Conversely, the oil content of soybean is generally negatively correlated with the use of fertilizer N (Sakla et al., 1988; Peric et al., 2009).

2.2. The Nitrogen Cycle

The nitrogen cycle is a dynamic system involving a variety of production and depletion pathways (Fig 2.2). Therefore, it is necessary to understand how different N sources are transformed into forms that are readily available for plant uptake. Nitrogen entering the soil—whether from fertilizer or natural sources—can be transferred to water and atmosphere via the

mechanisms of ammonia volatilization, nitrification, denitrification, and leaching (Fig 2.2). Nitrification and denitrification are two of the key processes that transform N in the soil. Nitrification occurs in a two-step process in which ammonia-oxidizing bacteria (AOB) and archaea (AOA) first convert NH_4^+ to NO_2^- (Eq 2.1) followed by the conversion of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Eq 2.2) (Lu et al., 2022). During the process of nitrification, the NO_3^- formed may be subjected to leaching, resulting in a decrease in N use efficiency (Ju & Zhang, 2017).

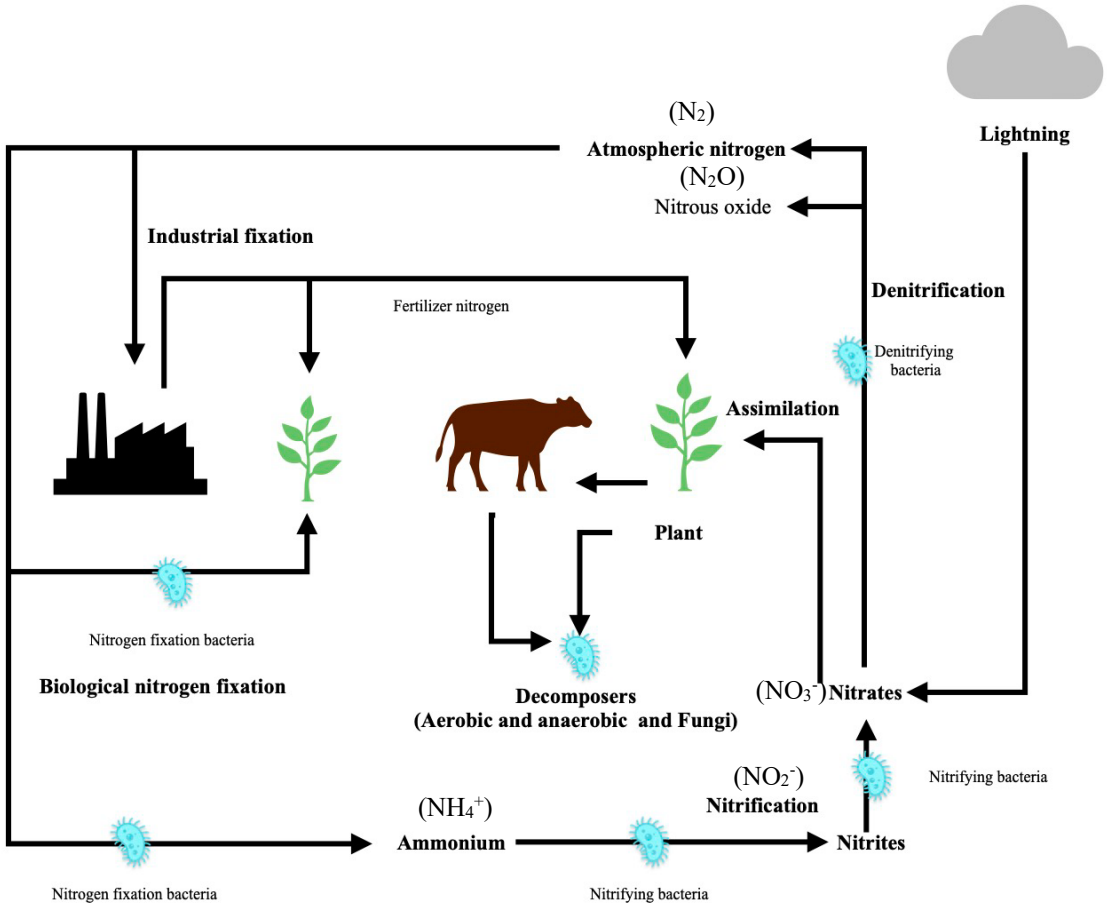

Fig. 2.2. The Nitrogen Cycle (adapted from IPNI, 2022).

$$2NH_4^+ + 3O_2 = 2NO_2^- + 2H_2O + 4H^+ + \text{Energy} \quad [2.1]$$

$$2NO_2^- + O_2 = 2NO_3^- + \text{Energy} \quad [2.2]$$

Denitrification occurs when the soil water content is high and soil microorganisms use NO_3^- as the terminal electron acceptor for respiration (IPNI, 2022). The end-product of the denitrification process depends on the overall environment, but one of the most significant products is nitrous oxide (N_2O)—a potent greenhouse gas (GHG). Nitrous oxide production can be significant in soils receiving high amounts of N fertilizer and especially under irrigation, which can result in anaerobic conditions, thereby creating a favourable environment for denitrifying bacteria and resulting in a high denitrification rate. This ultimately leads to an increased release of N_2O into the atmosphere and loss of available N (Qasim et al., 2022).

Ammonia volatilization occurs when NH_4^+ and urea-based fertilizers are applied to the soil and is a major pathway of N loss in agricultural systems—resulting in economic loss and reduced fertilizer N use efficiency (Pan et al., 2016). While other factors such as soil pH, soil moisture, soil texture, and environmental condition can also influence ammonia volatilization, the placement of the fertilizers is one of the key factors affecting volatilization of NH_4^+ and urea-based fertilizers. Volatilization losses can be especially significant in no-till systems where the N fertilizer is applied directly on the surface (IPNI, 2022).

To address issues associated with the impacts of excess N on the environment and human health, and to protect against economic loss, the agriculture industry has developed the 4R nutrient stewardship concept. The principles of 4R nutrient stewardship are Right source, Right time, Right rate, and Right place to optimize nutrient use efficiency and eliminate environmental risk. Although 100% N use efficiency (NUE) is unachievable, the main goal of using employing 4R nutrient management is to reduce unwanted losses of N fertilizer as much as possible.

One component of 4R nutrient stewardship is *Right Source*, which includes the use of enhanced efficiency fertilizers (EEFs) that include slow- or controlled-release products and products that use inhibitors that target specific pathways in the N cycle to better synchronized N delivery with crop demand (Trenkel, 2010). These products include urease inhibitors that retard urea hydrolysis to slow the accumulation of NH_4^+ in the soil, which protects against NH_3 volatilization and can slow nitrification and, in turn, denitrification (Olson-Rutz et al., 2011). Enhanced efficiency fertilizers employing nitrification inhibitors that slow the conversion of NH_4^+ to NO_3^- also are common (Norton & Ouyang, 2019). Depending on temperature, soil properties, and management factors (e.g., irrigation and fertilizer rate), research has shown that nitrification inhibitors can reduce NO_3^- leaching (~48%), N_2O emissions (~44%), and NO emission (~24%)

while increasing crop yield (~7.5%) and NUE (~12.9%) (Qiao et al., 2015; Norton & Ouyang, 2019).

In addition to EEFs, biological fertilizer products that involve N-fixing microorganisms have been gaining traction in the agriculture industry as they hold the potential to reduce the need for synthetic fertilizer N, enhance nutrient use efficiency, and mitigate environmental risks. Indeed, the use of microbial inoculants to optimize biological nitrogen fixation (BNF) in legumes has been a common practice for many decades (Ladha et al., 2022) and an effective *Rhizobium*-legume system can fix 20 to 300 kg N ha^{-1} yr^{-1} (Soumare et al., 2020) and account for 50 to 80% of the plant's N uptake (Liu et al., 2019). Whereas extending BNF to non-legumes has been slow to develop, commercial inoculants such as Envita™—which employs the non-symbiotic N_2-fixing bacterium *Gluconacetobacter diazotrophicus*—have been developed for use with non-legume crops such as rice (Filgueiras et al., 2019) and corn (Egamberdieva et al., 2021).

2.3. Biological Nitrogen Fixation (BNF)

Biological nitrogen fixation was discovered by Hellriegel and Wilfarth in 1886 when they determined that some legumes had the ability to use N_2 from the atmosphere as a nutrient source (Franche et al., 2009). Then in 1888, Dutch microbiologists isolated and identified the *Rhizobium leguminosarum* strain from root nodules (Robertson et al, 2011). Since then, more microbes with the ability to fix N have been discovered and by 1960 about a dozen genera of free-living N_2-fixing soil bacteria were known. Today, the development of molecular techniques for identifying microorganisms together with the use of ^{15}N-tracers to demonstrate N_2-fixing ability, has increased the number of know genera and species capable of BNF (Franche et al., 2009; Wagner, 2011).

The principle of BNF in legumes is straightforward; i.e., bacteria form a symbiotic relationship with a host plant to fix atmospheric N_2 and convert it to an available form that is then supplied to the host plant in exchange for carbohydrates and energy. However, the process itself is complex—requiring the enzyme nitrogenase—and very energy intensive (Eq 2.3):

$$N_2 + 8H^+ + 8e^- + 16\ ATP \xrightarrow[nitrogenase]{R.\ leguminosarum} 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i \qquad [2.3]$$

However, legume-rhizobia symbioses are species-specific; thus, to explore the advantages of BNF in non-legume crops, different types of N-fixing bacteria have been examined (Fig 2.3).

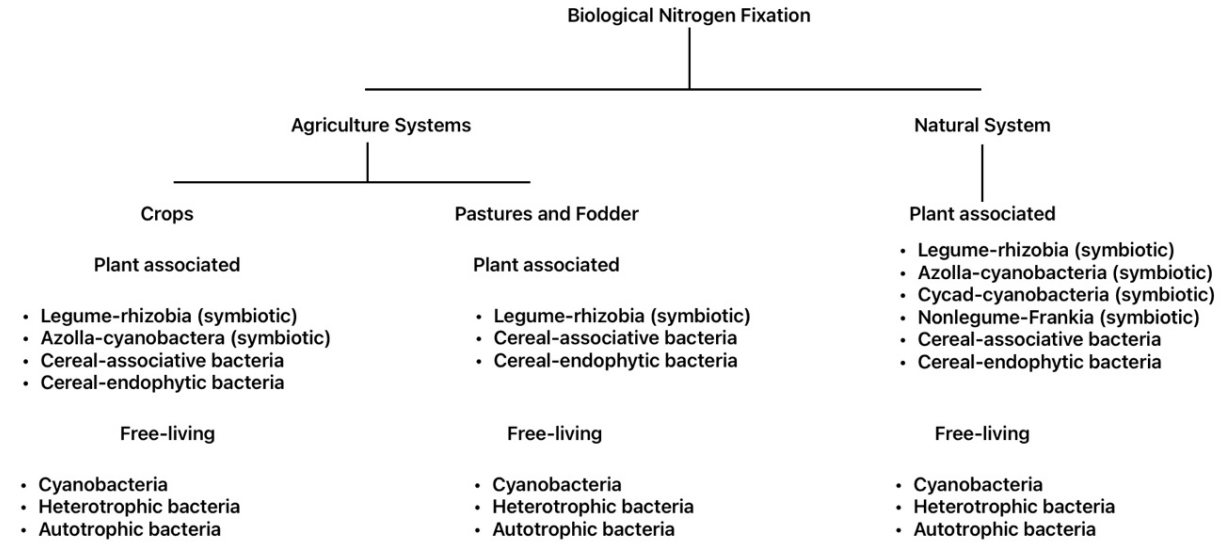

Fig. 2.3. Nitrogen-fixing organisms found in agricultural and natural systems (adopted from Wagner, 2011).

Among the different types of N_2-fixing bacteria, one of the more important is *G. diazotrophicus,* which is a gram-negative, non-spore forming, non-nodule producing, endophytic N-fixing bacterium that can survive in low pH, high sucrose, and high salt environments (Cavalcante & Dobereiner, 1988; Eskin et al., 2013; Chawla et al. 2014). In 2009, Bertalan et al. (2009) sequenced *G. diazotrophicus* Pal5—finding several characteristics related to N_2 fixation, plant growth promotion, sugar metabolism, the synthesis of auxin, and the occurrence of bacteriocins. This detailed sequencing of *G. diazotrophicus* provided new opportunities to manipulate plant-bacteria interactions with the aim of improving crop production and new biofertilizer development (Bertalan et al., 2009).

Interest in *G. diazotrophicus* originated from its ability to fix atmospheric N_2 in sugarcane in the absence of nodule formation. Unlike rhizobia, which target specific species and require the formation of nodules to fix atmospheric N_2, *G. diazotrophicus* is prokaryotic and an endophytic bacterium that can establish itself and colonize a host plant tissue while not exerting a harmful effect (Wu et al., 2021). The intercellular establishment of *G. diazotrophicus* starts in the root and as the plant grows, moves into the leaves where it can interact closely with the chloroplasts and possibly supply energy directly from the chloroplasts to *G. diazotrophicus* within the same cell (Dent & Cocking, 2017). Moreover, *G. diazotrophicus* has enzymes on its cell walls that can weaken the primary cell walls of the meristem allowing the bacterium to connect directly to the

plasma membrane of root cell protoplasts. As research has progressed, endophytic bacteria such as *G. diazotrophicus* have also shown potential to improve plant growth, promote resistance toward biotic and abiotic stress, and produce metabolites with medicinal potential (Wu et al., 2021; Döbereiner et al., 1993).

A significant effect of G. *diazotrophicus* colonization is plant growth promotion (PGP) associated with phytohormones such as Indole-3-acetic acid (IAA) and gibberellins A1(GA_1) and A3 (GA_3) (Bastián et al., 1998; Mehnaz & Lazarovits, 2006; Eskin et al., 2014)—all of which are critical to plant growth and development. Indeed, IAA is one of the most common auxin-class phytohormones that promote cell division, cell expansion, cell differentiation and fruit development (Bunsangiam et al., 2021) while GA_1 and GA_3 have important roles in controlling and promoting germination in cereal grains and other species (Gupta & Chakrabarty, 2013). In addition, *G. diazotrophicus* has been shown to (i) promote phosphorus and zinc solubilization (Mehnaz & Lazarovits, 2006; Eskin et al., 2014); (ii) induce defense mechanisms against the pathogen *Xanthomonas albilineans* in sugarcane (Arencibia et al., 2006); and (iii) express antifungal capacities against pathogens such as *Fusarium* spp. and *Helminthosporium* spp. (Mehnaz & Lazarovits, 2006).

A major benefit of *G. diazotrophicus* is that it is less species specific than *Rhizobium* spp.; for example, though *G. diazotrophicus* was originally discovered in sugarcane, subsequent studies found that it was also native to other plants such as sweet potato (*Ipomoea batats*), coffee (*Coffea arabica*), carrot (*Daucus carota*), and wetland rice (*Oryza sativa*) (Paula et al., 1991; Jimenez-Salgado et al., 1997; Madhaiyan et al., 2004; Muthukumarasamy et al., 2005). These discoveries prompted research to determine if the *G. diazotrophicus* could be developed into an inoculant for plants that did not natively contain the bacterium. The resulting discovery that high-value crops such as wheat, corn (*Zea mays*), tomato (*Lycopersicon eculentum*), oilseed rape (*Brassica napus*), and common bean (*Phaseolus vularis*) could be inoculated with *G. diazotrophicus* (Cocking et al., 2006; Trujillo- López et al., 2006), incentivized interest in its commercialization as a crop inoculant.

The various beneficial functions that *G. diazotrophicus* has been shown to provide notwithstanding, the driving force behind the development of *G. diazotrophicus* as a crop inoculant is its ability to produce the nitrogenase enzyme needed fix atmospheric N_2 (Fisher & Newton,

2005). Indeed, this nitrogenase consists of the catalytic molybdenum-iron protein (MoFeP), the iron protein (FeP), and several accessory proteins (Owens & Tezcan, 2018).

G. diazotrophicus lacks the nitrate reductase protein (Cavalcante & Dobereiner, 1988) and can tolerate up to 10 mM NO_3^- in liquid and semi-liquid growth media. Nevertheless, under certain circumstances *G. diazotrophicus* can be inhibited by high (> 5 mM) nitrate concentrations (Vessey & Pan, 2003; Medeiros et al., 2006). For example, under field conditions, growth inhibition of *G. diazotrophicus* was observed when a high level of N fertilizer was supplied (Eskin et al., 2014). Additionally, the *G. diazotrophicus* showed limited nitrogenase activity in the presence of ammonium-based fertilizer (Eskin et al., 2014). Therefore, unlike rhizobia, *G. diazotrophicus* can perform N_2 fixation in crops supplemented with nitrate-based fertilizers and even low amounts of ammonium-based fertilizer.

2.4. Verifying and Quantifying Plant Colonization by *Gluconacetobacter diazotrophicus*

Early attempts to verify the presence of *G. diazotrophicus* in host plants relied on PCR (Eskin et al., 2014). However, whereas simple PCR could identify the presence of *G. diazotrophicus* at high colony numbers, identification at low colony numbers required a nested PCR approach. Moreover, simple PCR was not capable of determining the number of the bacteria present. As a result, the most probable number (MPN) method was used to quantify *G. diazotrophicus* colonization (Eskin et al., 2014). The MPN method, however, was not considered to be particularly accurate and was eventually supplanted by enzyme-linked immunosorbent assay (ELISA) using species-specific polyclonal antibodies for *G. diazotrophicus* (da Silva-Froufe et al. 2009). The identification and quantification of *G. diazotrophicus* was later improved by using quantitative real-time polymerase chain reaction (RT-qPCR) (Galisa et al., 2012) using three genes (*rho, 23SrRNA* and *rpoD*) identified as stable reference genes in the RT-qPCR method. Compared to simple PCR, RT-qPCR is faster, more sensitive, and capable of simultaneously identifying and quantifying *G. diazotrophicus*.

More recently, a group of researchers from the National Research Council (NRC) of Canada in Saskatoon has developed a method utilizing droplet digital PCR (ddPCR) to identify and accurately quantify the number of *G. diazotrophicus* bacteria in plant samples. The ddPCR method was developed by Bio-Rad Laboratories, Inc. in 2011 to quantify nucleic acids (Hindson et al, 2011)—the fundamental concept being to divide the PCR reaction mastermix (comprising nucleic

acids, primers, and supermix) into approximately 20,000 microdroplets encased in oil to promote PCR amplification of individual template molecules. During endpoint reading via droplet flow cytometry, the supermix incorporates the DNA intercalating dye EvaGreen™ to differentiate positive droplets that contain a template molecule from negative droplets that lack the template molecule (Voegel et al., 2021). Afterward, the ddPCR software is used to calculate the number of template molecules in the sample by using Poisson statistics (Hindson et al, 2011). Compared to the earlier simple PCR and RT-qPCR methods, the advantage of ddPCR is absolute quantification and higher precision (Voegel et al., 2021).

2.5. Verifying N_2 Fixation in Plants

The stable isotopes of nitrogen (^{14}N and ^{15}N) are often used to study the fate of N in agricultural systems at scales ranging from microcosms to landscapes (Bedard-Haughn et al., 2003). The vast majority (99.6337%) of N has an atomic mass of 14 with only 0.3663% of the N having an atomic mass of 15 (Junk & Svec, 1958; Bedard-Haughn et al., 2003)—resulting in a *natural abundance* of ^{15}N of 0.3663 atom%. In general, however, the ^{15}N natural abundance of soils and plants is impacted by isotopic discrimination during mineralization, nitrification, denitrification, and leaching (Robinson, 2001; Brookshire et al., 2012) as well as by factors such as N fertilization and climate (Cheng et al., 2009). As a result, soils tend to be slightly ^{15}N enriched relative to the atmosphere. Nitrogen-15 natural abundance (NA) methods rely on these differences to track the flow of N in soils and plants (Warembourg, 1993). For example, plants that derive their N from the soil will have $\delta^{15}N$ values[1] similar to that of the soil N whereas plants that derive their N primarily through BNF will have $\delta^{15}N$ values closer to that of the atmosphere—reflecting dilution by *fixed N* that is ^{15}N depleted. Nitrogen-15 enrichment methods, on the other hand, rely on differences in atom% ^{15}N resulting from the addition of a ^{15}N-labelled N source (e.g., fertilizer, manure, or plant residues) to the soil.

One method for determining the amount of N derived from BNF is the $^{15}N_2$ reduction method (Warembourg, 1993). The basic principle of $^{15}N_2$ reduction method involves exposing the plant-inoculant system to a $^{15}N_2$ enriched atmosphere for a specific period of time, followed by

[1] Note: the isotopic abundance of ^{15}N is generally expressed as the ratio of ^{15}N to ^{14}N and is determined by measuring the difference between the isotopic ratio of a sample (R_{sample}) to that of a standard with a known isotopic composition ($R_{standard}$), with the difference reported in "delta (δ) notation".

^{15}N analyses of the labelled plant material (e.g., shoots, roots, and seeds) using isotope ratio mass spectrometry (IRMS). The percentage of total N that was fixed (*%Ndfa*) is then calculated using the equation:

$$\%Ndfa = \frac{^{15}N\ atom\%\ excess\ in\ sample}{^{15}N\ atom\%\ excess\ in\ atmosphere} \times 100 \qquad [2.4]$$

Isotope ratio mass spectrometry is widely used in archaeology, medicine, geology, biology, and agriculture due to its accuracy and precision in measuring variations in the abundance of isotopic ratios of light elements such as carbon ($^{13}C/^{12}C$), oxygen ($^{18}O/^{16}O$), hydrogen ($D/^{1}H$), nitrogen ($^{15}N/^{14}N$), and sulfur ($^{34}S/^{32}S$) (Muccio & Jackson, 2008). During measurement, the isotopic ratio of the sample is always compared to the isotopic ratio of an appropriate standard to eliminate bias and systematic error. Depending on the purpose of the study or the source material for the IRMS, different interfaces such as liquid chromatography (LC), elemental analyzer (EA), or gas chromatography (GC) can be used to introduce samples into the IRMS. The basic idea is to introduce the sample from a selected interface and ionize the samples inside the ionization source. Subsequently, ionized samples are accelerated to the magnetic analyzer and separated by magnet into different faraday detectors (Figure 2.4).

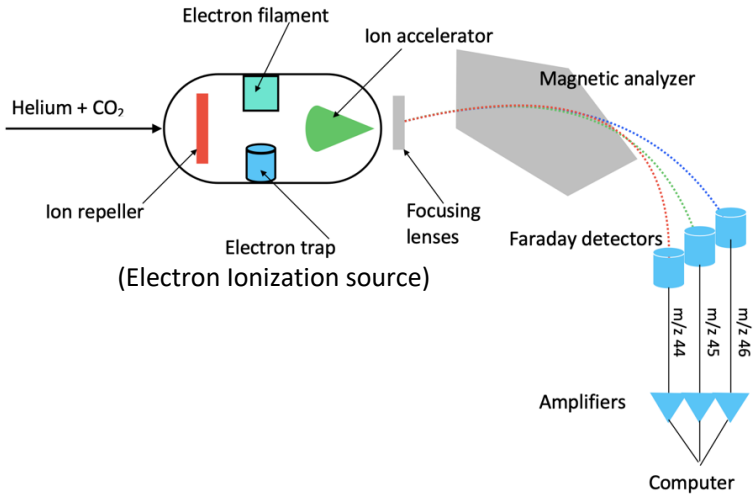

Fig. 2.4. Schematic of an isotope ratio mass spectrometer (adapted from Muccio & Jackson, 2008). The figure shows the basic components of an isotope ratio mass spectrometer needed for the quantification of natural abundance and enriched isotopes in soil and plant samples.

Finally, the ion detectors, such as faraday detectors at the end of the machine, read the signal and transfer it to a computer for processing and computation (Muccio & Jackson, 2008). Using IRMS and the $^{15}N_2$ reduction method Sevilla et al. (2011) were able to demonstrate conclusively that the N_2-fixing bacterial endophyte *Acetobacter diazotrophicus* was able to fix atmospheric N_2 and transfer the fixed N to sugarcane.

2.6. The Case for Biological N_2 Fixation in Non-legume Crops in Saskatchewan

Saskatchewan accounts for about 40% (24.4 Mha) of the total of cropland in Canada, with canola and spring wheat being the two most important crops—accounting for about 54% of total canola production and 44% of total spring wheat production in Canada in 2021 (Statistics Canada, 2022). To support this production, producers rely on chemical fertilizer to supplement the native fertility of the soil, with N being the primary limiting nutrient (other than water) and therefore the fertilizer required in greatest quantity (Anas et al., 2020). Indeed, between 2001 and 2021 N fertilizer consumption in Canada increased from 1.58 Mt to 2.73 Mt with Saskatchewan accounting for about 40% of the total N fertilizer used in Canada in 2021 (StatCan, 2023). Thus, if Saskatchewan producers are to meet the federal N_2O emissions reduction target set for 2030, more efficient use of, and alternatives to chemical fertilizers will be required. Recent advances in the development of microbial inoculants for BNF in non-legume crops may be help alleviate the need for chemical fertilizers and contribute to strategies designed to meet these emission reduction targets while protecting against yield loss.

3. MATERIALS AND METHODS

3.1 Inoculant

The inoculant used throughout this research was a commercial, food-grade, N_2-fixing bacteria (*Gluconacetobacter diazotrophicus*) marketed as Envita™ by Azotic North America Ltd. (Guelph, ON). Systemic N_2 fixation is achieved after the bacteria enter the plant to form a symbiotic relationship with the host and move throughout the plant as it grows. The inoculant used throughout this study was a pre-commercial dry powder concentrate of Envita™ that was prepared by Azotic Technologies Ltd. (Dunnington, UK).

The powdered inoculant was supplied in 10-g air-tight aluminum pouches and stored in a refrigerator at 4°C. After opening a pouch, the inoculant was divided into 0.20-g aliquots by weighing the inoculant into 2 mL microcentrifuge tubes that were then placed into 50-mL Falcon® tubes containing a small amount of silica gel desiccant (Sigma-Aldrich). The Falcon® tubes were then sealed with parafilm (PARAFILM®, USA) and stored in a refrigerator at 4°C until needed. All operations involving handling of the inoculant were conducted inside a Laminar air flow biocontrol cabinet (Thermal 1300 Series A2; Thermal Scientific).

3.2 Overview of the Study

The crops used in this study included the two major crops grown in Saskatchewan (spring wheat [*Triticum aestivum* L., var. ACC Wheatland] and canola [*Brassica napus* L., var. InVigor L233P]) and soybean (*Glycine max* L. Merr., var. AAC Halli). Wheat seed from Ardell Seeds Ltd. (Vanscoy, SK) was provided by Dr. J. Diane Knight, Department of Soil Science. Canola seed from BASF was provided by Dr. Jeff Schoenau, Department of Soil Science. Untreated soybean sourced from the breeding program of Dr. Elroy Cober (AAFC, Ottawa) and donated by Dr. Rosalind Bueckert (Crop Physiology), Department of Plant Sciences.

The initial approach to evaluating whether the *G. diazotrophicus* inoculant was fixing atmospheric N_2 and transferring it to the host plant (soybean and wheat) was to investigate the

process in a soil-less, semi-sterile, N-free environment (i.e., Leonard Jars). Canola could not be grown successfully in Leonard Jars and so was not included in these initial experiments. Subsequent experiments with all three plant species were conducted using soil-based systems. Different methods of applying the inoculant also were investigated: (i) direct seed inoculation (Sec. 3.3.1); (ii) inoculation at germination in a root bath (Sec. 3.3.2); and (iii) foliar application (Sec. 3.3.3). Regardless of growth medium or inoculation techniques, N_2-fixation was determined using $^{15}N_2$ labelling in a custom-made labelling chamber. All plant tissues were analyzed for total N and ^{15}N content using isotope ratio mass spectrometry (IRMS) as described in Sec. 3.7. A summary of the different experiments is presented in Table 3.1.

Table 3.1. Summary of the different experiments conducted. Plants were grown in Leonard Jars or in soil in pots. Details of the inoculation methods are outlined in Sec. 3.5. Plant tissues [shoot (SHT) and root (RT)] were analyzed for ^{15}N using IRMS and the presence of *G. diazotrophicus* (Sec. 3.7.1).

Plant	Plant tissue	Growth System	Inoculation method	Evaluated for ^{15}N	Evaluated for *Gd*
Wheat	SHT	Leonard jar	Direct seed	yes	no
Wheat	SHT	Leonard jar	Foliar	yes	no
Wheat	SHT	Soil	Direct seed	yes	no
Wheat	SHT	Soil	Foliar	yes	no
Wheat	SHT & RT	Soil	Direct seed	yes	yes
Wheat	SHT & RT	Soil	Root bath	yes	yes
Wheat	SHT & RT	Soil	Foliar	yes	yes
Soybean	SHT	Leonard jar	Direct seed	yes	no
Soybean	SHT	Leonard jar	Foliar	yes	no
Soybean	SHT & PODS	Soil	Direct seed	yes	no
Soybean	SHT & PODS	Soil	foliar	yes	no
Soybean	SHT & RT	Soil	Direct seed	yes	yes
Soybean	SHT & RT	Soil	Root bath	yes	yes
Soybean	SHT & RT	Soil	Foliar	yes	yes
Canola	SHT	Soil	Direct seed	yes	no
Canola	SHT	Soil	Foliar	yes	no
Canola	SHT & RT	Soil	Direct seed	yes	yes
Canola	SHT & RT	Soil	Root bath	yes	yes
Canola	SHT & RT	Soil	Foliar	yes	yes

In conjunction with this project, Azotic North America was working with Dr. Sean Hemmingsway (a molecular biologist) at NRC-Saskatoon who developed a protocol for confirming colonization of *G. diazotrophicus* in plant tissues using digital polymerase chain reaction technology (ddPCR; Appendix A). Plant tissue samples from several of the soil-based experiments (Table 3.1) were subsequently sent to Dr. Hemmingsway's lab for analysis.

3.3 Seed Treatments

Each experiment included three seed treatments: (i) no inoculant; (ii) Envita™; and (iii) heat-killed Envita™, with five replicates for each treatment. Initial experiments with soybean included an additional treatment; i.e., (iv) a commercial rhizobial inoculant (Cell-Tech®; NexusBioAg, Univar Canada, Ltd.)—included to verify that the pulse-labelling technique employed in these experiments was sufficient to determine whether $^{15}N_2$-fixation occurred. The heat-killed treatment provided a check to evaluate whether the Envita™ formulation (i.e., the matrix that the *G. diazotrophicus* is supported in) affected the ^{15}N signal independently of the inoculant organism. Heat-killed treatments involved application with Envita™ inoculant that had been autoclaved and cooled to room temperature. No-inoculant controls were treated with sterile, non-chlorinated deionized water (di-H_2O).

3.3.1 Seed sterilization protocols

Seed sterilization was performed to eliminate microbial contaminants such as fungi and bacteria that may have been present on the seed surface. Seed sterilization and seeding of the Leonard jars were performed in a laminar flow biocontrol cabinet. All seeds were initially rinsed in sterile di-H_2O. Each seed type required a slightly different sterilization protocol to achieve sterility without damaging the seed and affecting germination. Canola seed was surface sterilized by soaking in 30% H_2O_2 for 90 s followed by three 30-s rinses in sterile di-H_2O. Wheat seed was surface sterilized by soaking in 95% ethanol for 60 s, followed by a 10-min soak in a solution containing 5% NaOCl (commercial bleach solution) and 0.01% Triton X-100 (a non-ionic surfactant; Fisher Scientific, Waltham, MA). The bleach solution was decanted and discarded, and the seeds rinsed 5 times in sterile di-H_2O for 30-s per rinse. Soybean seed was sterilized using the same protocol as wheat except that the initial soak in ethanol was for 30 s followed by a 5-min

soak in 5% NaOCl – 0.01% Triton X-100 solution. The seeds were then rinsed 5 times in sterile di-H_2O for 30-s per rinse.

3.4 Plant Growth Systems

3.4.1 Leonard Jars

A Leonard jar is a semi-sterile, soil-less system for growing plants. It is essentially a small hydroponic unit in which the plant roots are anchored into a solid, inert medium (usually vermiculite) that is connected to a reservoir containing N-free nutrient solution through a cotton wick (Fig. 3.1). In my experiments, *ca*. 500 mL of pre-wetted vermiculite was used to fill the top, inverted bottle, and the neck of the bottle was plugged with a cotton ball to prevent the vermiculite from falling into the nutrient solution. The entire assembly was wrapped in foil and autoclaved, and after cooling the apparatus to room temperature, the bottom reservoir was filled with 400 mL of autoclaved dilute N-free nutrient solution (Somasegaran & Hoben, 1994). Filling the reservoir before autoclaving the entire apparatus resulted in unequal and unpredictable loss of the nutrient solution, hence the reason the reservoir was filled after autoclaving. Seeds or seedlings with an appropriate treatment were planted into the vermiculite and the Leonard jars placed in a controlled environment room with a 16h/8h day/night cycle and constant 22 °C day/night room temperature. The treatments were completely randomized and were re-randomized weekly.

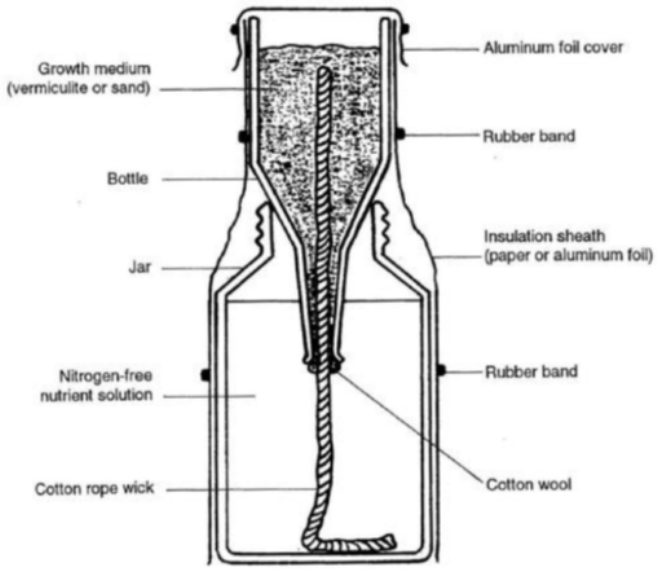

Fig. 3.1. Leonard jar assembly (from Somasegaran and Hoben, 1985).

3.4.2 Soil-based

Plants grown in soil tend to be more robust and healthier than those grown in Leonard Jars; thus, pot experiments were carried out after the Leonard Jar trials. All pot studies were conducted using a potting medium consisting of a 1:1 (w/w) mixture of sand and soil to facilitate root recovery. Surface soil (0–10 cm) was collected from the St. Denis National Wildlife Area (52°12' N 106°5' W) and brought to the Department of Soil Science where it was sieved to pass a 2-mm mesh screen, homogenized using Monarch soil mixer (Monarch Industries, Inc., Winnipeg, CA), air dried, and mixed (1:1 w/w) with silica sand (Gransuil; Covia, OH). The resulting sand:soil mix had a sandy loam texture (67.3% sand, 24.2% silt, and 8.6% clay), OC content of 1.76%, and was quite low in available nutrients: 2.2 mg NO_3-N kg^{-1}, 6.6 mg PO_4-P kg^{-1}, 298 mg K kg, and <3 mg SO_4-S kg^{-1}.

Approximately 450 g of the sand:soil mix was weighed into 10-cm diameter pots. To minimize evaporation from the pots, a layer of small, white plastic beads was placed on the surface of the soil. Nevertheless, to maintain soil moisture near 70% water holding capacity (WHC) it was necessary to water the pots twice per day (i.e., at 09:00 and 16:00 h). An autoclaved dilute N-free nutrient solution (Somasegaran & Hoben, 1994) was applied once per week during a regular morning watering event. Plants were grown in a Conviron® controlled environment chamber with a 16h/8h day/night cycle with 28/22°C day/night temperatures. Treatment pots were completely randomized in the growth chamber and were re-randomized weekly.

3.5 Seed Inoculation Protocols

All operations involving preparation and handling of the inoculant were conducted inside a Laminar air flow biocontrol cabinet.

3.5.1 Reconstitution of the *G. diazotrophicus* inoculant

Prior to inoculation the *G. diazotrophicus* was reconstituted by mixing 0.20 g of the dry Envita™ product (10^{15} cfu g^{-1}) with 2 L of non-chlorinated di-H_2O (pH between 4 and 7.5) in an Erlenmeyer flask sealed with Parafilm that had been disinfected with 70% ethanol. The solution was mixed at room temperature for 30 min using a magnetic stirrer. The solution was then refrigerated and used within 72 h. The reconstituted *G. diazotrophicus* yielded *ca.* 10^{11} cfu mL^{-1}.

3.5.2 Direct seed application (DSA)

Surface sterilized seed (5 g) was placed into a sterile Whirl-Pak® bag containing enough reconstituted *G. diazotrophicus* (10–20 mL, depending on seed size) to ensure that all seeds were in contact with the inoculant. The seeds were soaked for 10 min, after which the excess inoculant solution was decanted and discarded, and the seed transferred to disposable sterile petri-dishes (100 mm × 5 mm) fitted with a Whatman # 2 filter paper that was moistened with an additional 10 mL of inoculant. The seeds were incubated at room temperature in the dark overnight before being seeded (five seeds per jar or pot) into the vermiculite chamber of a Leonard jar or into a pot with soil (see Sec. 3.4).

3.5.3 Root bath application (RBA)

Seeds (10 to 20, depending on plant species) were germinated on sterile Whatman #2 filter paper fitted into a disposable sterile petri-dish (100 mm × 5 mm) and moistened with 10 mL of the reconstituted *G. diazotrophicus*. The petri dishes were sealed with parafilm and stored in a controlled temperature chamber at 28°C in the dark for five to seven days. The filter paper was remoistened with the reconstituted *G. diazotrophicus* solution as needed to prevent drying. Five inoculated seedlings were then transplanted into the sand:soil mixture (see Sec. 3.4.2). To avoid washing the inoculant from the roots, no water was applied to the pots during the first three days after transplanting—after which the normal watering cycle was maintained (see Sec. 3.4.2).

3.5.4 Foliar application

The *G. diazotrophicus* was reconstituted as described above, except that 0.10 g of the powder was mixed with 1.0 L sterile, non-chlorinated, distilled, deionized water containing 1.0 mL of the non-ionic surfactant Silwet L-77 (PlantMedia, bioWorld, Dublin, OH) and transferred to a sterile spray bottle. The test crops were seeded (n = 5) into the vermiculite chamber of a Leonard jar (see Sec. 3.4.1) or a pot containing the sand:soil mixture maintained at *ca*. 70% WHC with sterile diH_2O (see Sec. 3.4.2). The inoculant solution was sprayed onto the leaves approximately two weeks after planting; i.e., at the 2nd true leaf stage for wheat, the V2 to V4 stage (2 to 4 leaves fully developed) for soybean, and the BBCH 12 to 14 stage (2 to 4 leaves unfolded) for canola.

3.6 Pulse Labelling with $^{15}N_2$

Pulse labelling with $^{15}N_2$ began 21 days after germination (DAG) when the plants were placed inside an air-tight chamber designed and built specifically for this study (Fig. 3.2). The dimensions of the labelling chamber were 90 cm × 60 cm × 60 cm (h×w×d), yielding an internal volume of 324 L. Styrofoam inserts (5 cm × 60 cm × 50 cm; n = 7) were installed in the chamber to reduce the headspace volume—thus reducing the amount of $^{15}N_2$ gas required—and could be removed to accommodate plant growth. The chamber was connected to an expandable bladder (24 cm × 30 cm; Ready Containment; Palmetto, FL) to accommodate changes in atmospheric pressure via a 3-way valve fitted with a port for injecting $^{15}N_2$ into the chamber. The CO_2 concentration in the chamber was maintained between 450 and 600 ppmV using an Autopilot CO_2 monitor and controller (Hydrofarm APC8200; Petaluma, CA) connected to a small (15 L) tank of CO_2. In addition, the chamber was fitted with fan for air circulation, an air conditioning unit (Hydrofarm AACH25HP; Petaluma, CA) to cool the air temperature inside the chamber during a labelling event (i.e., to 28°C during the day and 25°C during the night), four LED lights (XLamp® CXB3590; Cree LED, Newark, CA) and a light timer (Intermatic HB880R; Chicago, IL) to regulate the day/night cycle within the chamber.

At 21 DAG—and during each subsequent labelling event—the plants were moved into the pulse labelling chamber and the chamber sealed and powered up, which automatically turned on the lights, CO_2 monitor and controller, timer for day/night cycle, and air conditioning unit. Once the chamber environment had stabilized, 3000 mL of 99.5% of $^{15}N_2$ was injected into the chamber using 140-mL and 60-mL syringes, and the time of the last of $^{15}N_2$ injection recorded. Each labelling event lasted 48 h after which the plants were removed from the labelling chamber and returned to the Conviron® controlled environment chamber. Pulse labelling was repeated at 28 and 35 DAG and the plants destructively harvested 48 h after the last labelling event.

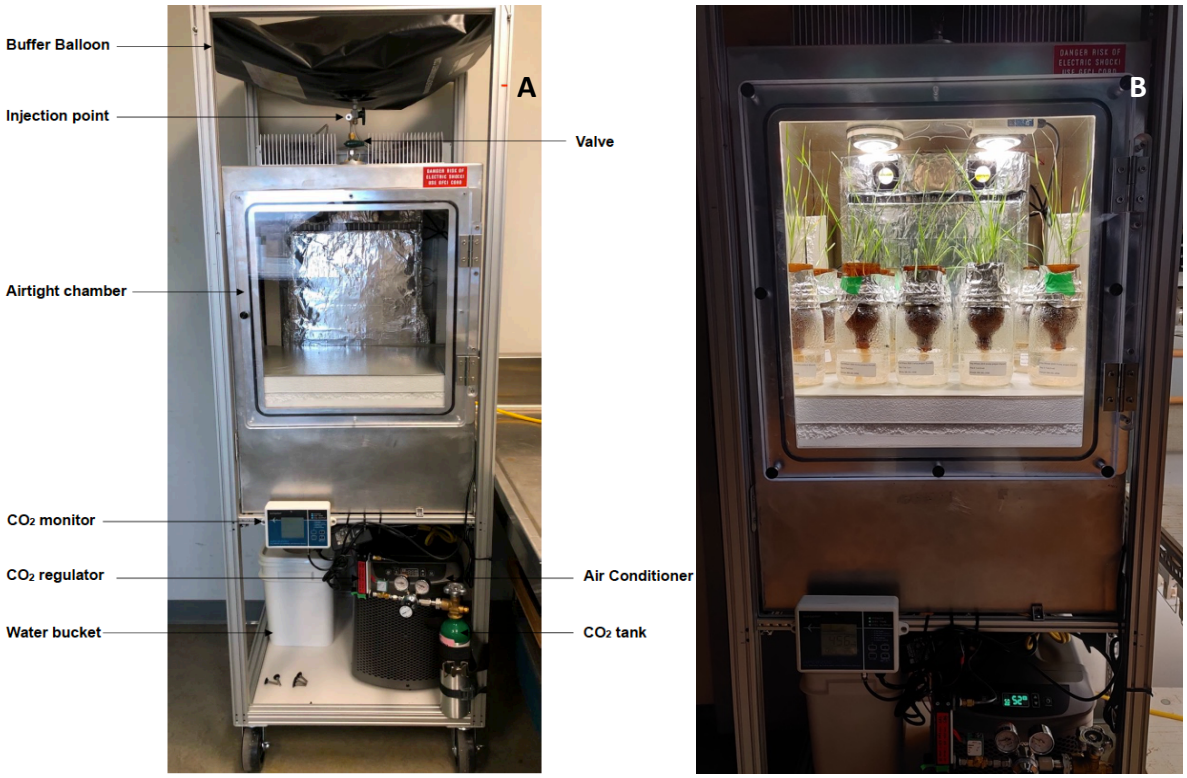

Fig. 3.2. The pulse labelling chamber (A) empty, showing the components of the system, and (B) containing wheat plants in Leonard Jars during a $^{15}N_2$ labelling event.

3.7 Plant Sampling and Processing

Plants were separated into shoots and roots and the roots washed free of the rooting medium. In early experiments only the shoots were analysed, while in later experiments the shoots and roots were analysed separately. As well, in one experiment pods with developing seed were separated from the shoots of soybean and analysed for ^{15}N content separately. All plant tissues were oven-dried to a constant weight at 60°C in a forced-air oven and the dry biomass recorded. These dried tissues were ground using a Wiley Mill and then further ground in a ball mill. Subsamples of the dried, ground shoot and root samples were encapsulated in tin capsules and analyzed for ^{15}N content (atom% ^{15}N) and N concentration (%N) on a Costech ECS4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA) coupled to a high-precision (0.06‰ for $\delta^{15}N$) Delta V mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chickpea flour with an atom%^{15}N = 0.3691 was used as a lab reference.

3.7.1 Droplet digital polymerase chain reaction (ddPCR analysis)

While the $^{15}N_2$ labelling study was underway, Azotic North America Ltd. contracted Dr. Sean Hemmingsway at NRC-Saskatoon to develop a protocol to verify colonization of *G. diazotrophicus* in plant tissues using droplet digital polymerase chain reaction (ddPCR) technology. The ddPCR procedure provides a sensitive, precise, and accurate method for amplifying sequences of different nucleic acids, including specific nucleic acid segments that are unique to *G. diazotrophicus*. The method quantifies the number of copies of the target sequence found in the plant tissues—with the data interpreted as follows: negative (<5 copies), borderline (5-8 copies), and positive (>8 copies). Because of the requirement for fresh shoot and root tissues, only experiments conducted during the latter stages of the study were sampled and analysed using ddPCR. That is, tissues from the earlier studies could not be analysed because they had been dried.

The protocol for confirming colonization of the plant tissues by *G. diazotrophicus* using ddPCR is described in Appendix A. Briefly, at harvest following the last $^{15}N_2$ pulse labelling event a subsample of fresh tissue was collected and transported on ice to the Hemmingsway lab where the materials were freeze dried in preparation for the ddPCR analysis. Liquid N_2 was not used in the freeze-drying process to avoid any contamination/alteration of the ^{15}N signals in the test tissues.

3.8 Statistical Analysis

The experiment was set up using a completely randomized design, and all statistical analyses were conducted using CoStat ver. 6.451 (Cohort Software, 2017). To obtain a normal distribution and homogeneity of variance, data transformations were performed when necessary. Afterward, data were back-transformed for reporting and presentation. Most atom%^{15}N data were transformed using the binary logarithm transformation to reduce heterogeneity of variance and obtain a normal distribution. However, for soybean the data transformation did not result in a normal distribution; thus, the nonparametric Kruskal-Wallis test was used to assess treatment differences for soybean. Atom%^{15}N values for plants inoculated with the heat killed Envita™ were never different from the controls and as such are not reported. Biomass data was subjected to ANOVA using a one-way analysis for treatment ($\alpha = 0.05$) and means comparisons were conducted using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$). Means comparisons for data with missing values were conducted using the least significant difference (LSD) test ($\alpha = 0.05$).

4. RESULTS

4.1 Leonard Jar Study

Whereas wheat and soybean were successfully grown in Leonard jars, canola growth was extremely poor and as a result the canola plants were not used in the ^{15}N-labelling study. Wheat was initially inoculated with *G. diazotrophicus* (*Gd*) by applying the inoculant directly to the seed; however, this resulted in no significant increase in the ^{15}N content of the wheat shoots relative to that of the non-inoculated control plants (Table 4.1). Thus, a second experiment was established with the wheat plants receiving a foliar application of the Envita™ inoculant. Although these plants exhibited about a 3-fold increase in total N uptake, the ^{15}N content of the treatment group (0.3680 atom%) did not differ from that of the non-inoculated control plants (0.3675 atom%) (Table 4.1).

Table 4.1. Total and ^{15}N content of wheat, canola, and soybean grown in Leonard jars and pulse-labelled with $^{15}N_2$.

Crop	Inoculant	Mode of application	Total N (%)	^{15}N (atom%) [a]
Wheat	Non-inoculated control	- - -	0.21	0.3675 a
	G. diazotrophicus	Direct to seed	0.21	0.3680 a
Wheat	Non-inoculated control	- - -	0.76	0.3675 a
	G. diazotrophicus	Foliar	0.72	0.3673 a
Soybean	Non-inoculated control	- - -	2.06	0.3683 b
	B. japonicum	Direct to seed [b]	3.13	0.6345 a
	G. diazotrophicus	Direct to seed	2.56	0.3696 b
	G. diazotrophicus	Foliar	2.16	0.3935 b

[a] Within columns, and for a given crop, means followed by the same letter are not significantly different according to $LSD_{0.05}$.

[b] The roots of plants inoculated with a commercial strain of *Bradyrhizobium japonicum* developed nodules indicating that these plants were capable of fixing atmospheric N_2.

As a positive control, soybean was inoculated with a commercial strain of *B. japonicum* (Cell-Tech®) using the direct seed application method. At harvest, nodules on the roots of these

plants were observed in two of the three control jars and, on average, these plants were found to contain significantly elevated concentrations of ^{15}N (0.6345 atom%) compared to the non-inoculated control plants (0.3683 atom%) (Table 4.1). Shoots of the soybean receiving a foliar application of the Envita™ inoculant contained about 7% more ^{15}N than either the non-inoculated controls or the plants inoculated with *Gd* using the direct seed application method (Table 4.1), though the increase was not significant.

4.2 Pot Study - Soybean

Following the Leonard Jar study, soybean were grown in 1:1 (w/w) sand:soil mix in a pot study and in addition to being analyzed for total and ^{15}N content, the presence of *G. diazotrophicus* in the plant tissues was determined using ddPCR (see Sec. 3.7.1). At harvest the plants were separated into shoots and roots and it was observed that the roots were nodule free, indicating that there were no native rhizobia in the sand:soil mix. In general, the presence of *G. diazotrophicus* in the shoots and roots was greatest (i.e., *Gd* index = 1) when the inoculant was applied as a foliar or root bath treatment (Table 4.2). However, only when the inoculant was applied as a root bath did it affect the ^{15}N content of the shoots (Table 4.2).

Table 4.2. Nitrogen (total and ^{15}N) content and *Gd* inoculation status of soybean grown in a 1:1 (w/w) sand:soil mix and pulse-labelled with $^{15}N_2$.

Treatment/Mode of application	Biomass (g)	Total N (%)	^{15}N (atom%)	*Gd* index[a]
		Shoots		
Non-inoculated control	2.61 a	1.40 a	0.3698	-0.4
Direct to seed	2.63 a	1.49 a	0.3808	-0.4
Foliar	2.34 a	1.61 a	0.3773	1.0
Root bath	1.56 b	1.40 a	0.3917	1.0
		Roots		
Non-inoculated control	0.51 a	1.85 a	0.3698	-0.8
Direct to seed	0.50 a	1.77 ab	0.3745	0.6
Foliar	0.45 ab	1.94 a	0.3725	1.0
Root bath	0.36 b	1.42 b	0.3836	1.0

[a] For each plant component (shoots and roots) and within columns, means followed by the same letter are not significantly different according to Tukey $HSD_{0.05}$

[b] Each sample was rated as positive (+1), borderline (0), or negative (-1) for the presence of *G. diazotrophicus*. The values for the five replicate samples were then averaged to yield an *Gd* index ranging from -1.0 to 1.0, with a value of -1.0 indicating that all five samples tested negative for the detection of *G. diazotrophicus* and a value of 1.0 indicating that all five samples tested positive.

Tissues from the non-inoculated soybean controls were expected to test negative for *Gd*; however, only two of the five reps of tested negative, with the shoots from the other three reps yielding borderline detections (Table A.1). Additionally, root samples from the soybean controls yielded four reps that tested negative for *Gd* and one borderline G.d detection (Table A.2). It is likely that the presence of small numbers of *Gd* in the control samples resulted from contamination during application of the foliar treatment.

Direct seed application (DSA) treatments yielded results that differed from those of the foliar and root bath treatments. Overall, there was poor colonization of the shoots of the DSA plants, with only one sample testing positive for *Gd*, one with a borderline positive test, and three testing negative (Table 4.2). Furthermore, although on average the shoot samples yielded a small increase in ^{15}N content compared to the control samples, this increase was not significant. Unsurprisingly, DSA of the Envita™ inoculant had no significant effect on shoot biomass or total N content. In contrast, *Gd* colonization of the roots was generally good, with 4 of 5 samples testing positive. Nevertheless, inoculation yielded no significant ^{15}N enrichment of the root tissues (Table 4.2).

Foliar application of the Envita™ inoculant was considerably more successful, with all five reps testing positive for *Gd* in both the shoots and roots. On average, however, the number of *Gd* copies in the shoot samples (3400 copies 100-ng^{-1} DNA) was 170-times greater than that of the root samples (20 copies 100-ng^{-1} DNA) (Table A2). But despite significant colonization of the soybean shoots, neither the shoot biomass, total N, nor ^{15}N content of the plant tissues differed significantly from those of the control plants (Table 4.2). Similar results were observed for the root samples, though the foliar application did result in a 12% decrease in root biomass.

Similar to the foliar treatment, root bath application (RBA) of the Envita™ inoculant yielded positive *G.d* tests in the shoots and roots of all five test plants (Table 4.2). In this case, however, the average number of *Gd* copies in the root tissues (718 copies 100-ng^{-1} DNA) was greater than that of the shoot tissues (434 copies 100-ng^{-1} DNA). Again, however, the shoot and root tissues of plants inoculated using the RBA technique exhibited ^{15}N enrichments that on average were comparable to those of the control plants (Table 4.2)—with one exception (see Table A2). That is, the tissues of one plant exhibited significantly higher enrichments in both the shoots (0.4772 atom% ^{15}N) and roots (0.4373 atom% ^{15}N). Surprisingly, the RBA treatment yielded plants that were much smaller than either the control plants or the plants receiving DSA or foliar

treatments—with significant reductions in shoot and root biomass of 59% and 36%, respectively (Table 4.2).

4.3 Pot Study - Wheat

Wheat plants grown in a 1:1 (w/w) sand:soil mix in pots were inoculated with Envita™ using DSA, foliar, and RBA methods and labelled using $^{15}N_2$ enrichment. After harvest, shoot and root tissues from the plants were analyzed for N content (both total and ^{15}N) and the presence of *G. diazotrophicus*. Tissues samples from the control (non-inoculated) plants all tested negative for the presence of *G.d* in both the shoots and roots (Table 4.3). Moreover, the ^{15}N content of the shoots (0.3712 atom%) and roots (0.3707 atom%) was comparable to that of the soybean controls (i.e., 0.3698 atom% ^{15}N for both the shoot and root samples) (Table 4.2).

Table 4.3. Nitrogen (total and ^{15}N) content and *Gd* inoculation status of wheat grown in a 1:1 (w/w) sand:soil mix and pulse-labelled with $^{15}N_2$.

Treatment/Mode of application	Biomass (g)	Total N (%)	^{15}N (atom%) [a]	*Gd* index [b]
		Shoots		
Non-inoculated control	0.54 b	1.76 a	0.3712 b	-1.0
Direct to seed	0.52 b	1.74 a	0.3713 b	-1.0
Foliar	0.61 b	1.60 b	0.3712 b	0.2
Root bath	0.37 a	1.68 ab	0.3718 a	-1.0
		Roots		
Non-inoculated control	0.18 a	1.26 a	0.3707 b	-1.0
Direct to seed	0.17 a	1.25 a	0.3708 b	1.0
Foliar	0.22 a	1.15 a	0.3708 b	-0.6
Root bath	0.22 a	1.30 a	0.3711 a	1.0

[a] For each plant component (shoots and roots) and within columns, means followed by the same letter are not significantly different according to Tukey $HSD_{0.05}$

[b] Each sample was rated as positive (+1), borderline (0), or negative (-1) for the presence of *G. diazotrophicus*. The values for the five replicate samples were then averaged to yield an *Gd* index ranging from -1.0 to 1.0, with a value of -1.0 indicating that all five samples tested negative for the detection of *G. diazotrophicus* and a value of 1.0 indicating that all five samples tested positive.

Direct seed application the Envita™ inoculant yielded positive tests for *Gd* in all the root samples (Table 4.3), with copy numbers of *Gd* ranging from 11 to 4090 copies per 100-ng DNA (median value = 39 copies per 100-ng DNA) (Table A.3). There was, however, no ^{15}N enrichment

of the root tissues (Table 4.3). Foliar application of the Envita™ inoculant yielded three positive *Gd* tests and two negative tests for the shoots; but again, *Gd* copy numbers were low (averaging *ca*. 10 copies 100-ng DNA) and there was no ^{15}N enrichment of the shoots. The foliar application failed to produce a positive *Gd* index for the roots—with only two plants yielding borderline positive tests for *Gd*—and inoculation had no significant effect on the ^{15}N content of the roots (Table 4.3).

Root bath application of the Envita™ inoculant resulted in positive *Gd* tests for the root samples from all five wheat reps—with *Gd* copy numbers ranging from 136 to 4362 (median value = 816 copies per 100-ng DNA). Conversely, shoot samples from all five reps tested negative for *Gd* (Tables 4.3 and A3). The RBA treatment of the wheat yielded smaller plants than the control treatment—with an average shoot biomass that was 31% lower than that of the control plants. However, this did not affect the total N content of the shoots, which also exhibited a small but significant increase in ^{15}N content (Table 4.3). Likewise, the RBA treatment had no significant impact on either root biomass or total N, though it did produce a small but significant increase in ^{15}N content relative to the control plants.

4.4 Pot Study - Canola

Canola plants inoculated with Envita™ and grown in a 1:1 (w/w) sand:soil mix in pots were labelled using the $^{15}N_2$ reduction technique (Sec. 3.6.1). After harvest, shoot and root tissues from the plants were analyzed for N content (both total and ^{15}N) and the presence of *G. diazotrophicus*. Surprisingly, four of the five shoot samples from the controls tested positive for *Gd* (Table 4.4), though the copy numbers were low (i.e., ≤ 11 copies per 100-ng DNA) (Table A4). At the same time, the root samples all tested negative for *Gd* (Table 4.4).

Foliar application of Envita™ to the canola resulted in positive tests for *Gd* in all the shoot samples but yielded inconsistent results in the root samples (Table 4.4) with three positive and one borderline positive test, and one negative test (Table A4). In addition, whereas *Gd* copy numbers in the shoot samples averaged 4572 copies per 100-ng DNA, root samples with a positive test result averaged only 8 copies per 100-ng DNA (Table A.4). Regardless, there was no difference in the ^{15}N content of the inoculated shoots and roots compared to the control plants (Table 4.4) and only a small, but not significant increase in shoot and root biomass (i.e., 13% and 9%, respectively).

Direct seed application of the Envita™ resulted in positive tests for *Gd* in 4 of 5 shoot samples but produced only two borderline positive tests in the root samples (Table A4). Despite the positive tests for *Gd*, however, the ^{15}N concentrations in the shoots (0.3723 atom%) and roots (0.3709 atom%) did not differ from those in the control plants (Table 4.4). Similar to the foliar treatment, biomass and total N content from the shoot and root sample had slightly increase and decrease but no significant change statistically.

Table 4.4. Nitrogen (total and ^{15}N) content and *Gd* inoculation status of canola grown in a 1:1 (w/w) sand:soil mix and pulse-labelled with $^{15}N_2$.

Treatment/Mode of application	Biomass (g)	Total N (%)	^{15}N (atom%) [a]	*Gd* index [b]
		Shoots		
Non-inoculated control	0.46 b	1.05 a	0.3724 b	0.6
Direct to seed	0.49 b	0.99 a	0.3723 b	0.8
Foliar	0.52 b	1.07 a	0.3723 b	1
Root bath	0.35 a	1.08 a	0.3731 a	0
		Roots		
Non-inoculated control	0.11 a	1.71 ab	0.3710 b	-1
Direct to seed	0.10 a	1.75 ab	0.3709 b	-0.6
Foliar	0.12 a	1.84 a	0.3710 b	0.4
Root bath	0.10 a	1.60 b	0.3716 a	-0.4

[a] Each sample was rated as positive (+1), borderline (0), or negative (-1) for the presence of *G. diazotrophicus*. The values for the five replicate samples were then averaged to yield an *Gd* index ranging from -1 to 1, with a value of -1 indicating that all five samples tested negative for the detection of *G. diazotrophicus* and a value of 1 indicating that all five samples tested positive.

[b] Within columns, means followed by the same letter are not significantly difference according to Tukey $HSD_{0.05}$

Canola plants that received a root bath application (RBA) of Envita™ produced shoots that yielded two positive, 1 borderline positive, and two negative tests for *Gd* (Table A.4). On average, the ^{15}N content of the shoots was marginally greater ($P \leq 0.05$) than that of the control plants (i.e., 0.3731 atom% *vs* 0.3724 atom%). At the same time, the root samples yielded 1 positive and one borderline positive test for *Gd* with three samples testing negative. And like the shoots, the average ^{15}N content of the roots was slightly greater ($P \leq 0.05$) than that of the control plants (i.e., 0.3716 atom% *vs* 0.3710 atom%). In both the shoots and roots, however, the small increase in ^{15}N did not translate into an increase in either total N content or biomass production (Table 4.4).

5. DISCUSSION

To verify the BNF ability of the Envita™, the study focused on two criteria: (i) did the plants treated with Envita™ test positive for the presence of *G. diazotrophicus* in their shoot and root tissues, and (ii) did the shoot and/or root tissues of the treated plants yield a significant increase in ^{15}N content relative to the control (untreated) plants. For a crop treatment to be considered successful, it would need to fulfill both criteria.

The efficacy of the pulse labelling procedure (see Sec. 3.6) was first evaluated by placing soybean plants inoculated with a commercial *B. japonicum* inoculant in the labelling chamber at 21 DAG, injecting $^{15}N_2$ enriched air into the chamber, and removing the plants from the labelling chamber after 48 h. The $^{15}N_2$ labelling was then repeated at 28 and 35 DAG before the plants were harvested and analysed for ^{15}N content using IRMS. Plant tissues from the *B. japonicum*-inoculated soybean were found to contain 1.7-times more ^{15}N than the control plants, confirming the effectiveness of the labelling procedure.

Leonard jar experiments were conducted during the initial stage of this research using a liquid form of the inoculant provided by Azotic Technologies Ltd. (Dunnington, UK; the parent company of Azotic North America). The Leonard jars are a semi-sterile system in that the system is sterile at the time of seeding, but after seeding the jars are placed in a non-sterile environmental chamber and are subject to infection by air-borne contaminants. However, they are free of 'natural' soil organisms and thus do not undergo competition for resources with other soil organisms. Furthermore, the Leonard jar systems are N free, except for being exposed to N_2 in the ambient air and $^{15}N_2$ in the atmosphere created during pulse labelling. Consequently, any ^{15}N found in the plant tissues in excess of that in the control plants must have derived from BNF of the $^{15}N_2$ enriched atmosphere.

Early trials with wheat and soybean grown in Leonard Jars revealed no BNF occurring in the wheat when the inoculant was applied directly to the seed or as a foliar inoculant. However, high enrichment in two of five soybean plants in Leonard jars (i.e., 1.7-times that of the control plants)—together with the observation that root nodules were not present—provided clear

evidence of BNF by the introduced *G. diazotrophicus* and its subsequent transfer to the soybean tissues. The small amount of enrichment in the soybean inoculated using the DSA method was insufficient to conclusively indicate BNF—and even if BNF was occurring the amount of N acquired through this pathway was too small to be of practical importance. Overall, the results of the Leonard Jar study suggested that (i) the Envita™ inoculant was capable of BNF in soybean, and (ii) that successful inoculation was dependent on the method of application and was difficult to achieve on a consistent basis. In addition, the Leonard Jars proved to be unsuitable for growing canola; consequently, the research approach shifted to a pot study with a sand:soil mix to ensure that all three target crops could be tested.

In general, plants grown in the sand:soil mix in pots were more robust and visibly healthier than those grown in Leonard Jars. Although the sand:soil mix had a low N content (2.2 mg kg^{-1} available N), in the absence of active BNF the plants could still access N via root uptake. The first set of trials in the soil systems examined ^{15}N incorporation into shoots when the Envita™ inoculant was applied either as a DSA or a foliar application. Neither wheat, canola nor soybean showed any indication of BNF, in that there was no ^{15}N enrichment of the plants. The Leonard jar experiments indicated that successful foliar inoculation is possible, but these initial soil experiments demonstrated that actually introducing the *G. diazotrophicus* organism to colonize the target plant tissues is challenging. For example, compared to the Leonard jar system, soils have the added challenge of native microbial populations competing with the introduced organisms (Hibbing et al., 2010). Consequently, a second set of pot studies with the sand:soil mix was expanded to include a RBA that involved soaking the germinating seed in liquid inoculant and then transplanting the seedling into the sand:soil mix (see Sec. 3.5.3). This second set of experiments also included an evaluation of the shoot and root tissues for the presence of *G. diazotrophicus* via the ddPCR method at NRC-Saskatoon (section 3.7.1).

While previous researchers have identified the mechanisms and demonstrated the beneficial effects of inoculating *G. diazotrophicus* into crops such as rice, wheat, sorghum, and corn, the methods of inoculation, population, and growth medium can vary (Tian et al., 2009; Luna et al., 2010; Ahlawat et al., 2021). Initially, the target crops were rice and corn with an in-furrow or foliar application of the inoculant (O'Callaghan et al., 2021); however, in these studies the presence of *G. diazotrophicus* in the plant tissues was not confirmed. In other research, *G. diazotrophicus* was inoculated into corn roots using an RBA method (Tian et al., 2009) similar to

the one used in the present study. In addition, the authors used the PCR method to confirm the presence of *G. diazotrophicus* within the plant tissues and found that inoculation success varied considerably—averaging 34 ± 30% in the roots, 28 ± 26% in the stems, and 16 ± 18% in the leaves of the 17 grain corn genotypes (totaling 347 plants) inoculated. Importantly, this study established that *G. diazotrophicus* could not only establish itself in the roots but that it could then move to other plant tissues presumably via the xylem. In the present study, *G. diazotrophicus* was detected in 25 of 45 shoot samples (i.e., 56%) and in 28 of 45 root samples (i.e., 62%), with soybean exhibiting the highest and most consistent success rate; i.e., 73% of shoots and 93% of roots tested positive for the presence of *G. diazotrophicus*. However, populations of *G. diazotrophicus* in the roots of the soybean were about two orders of magnitude lower than those in the shoots and while there was a weak positive correlation between *G. diazotrophicus* numbers and atom% ^{15}N in the shoots ($r_s = 0.502$; $P = 0.057$) there was no such correlation for the root samples ($r_s = -0.054$; $P = 0.850$). Interestingly, the total N content of roots from the soybean plants receiving the RBA treatment were all lower than in the roots of the control (non-inoculated) plants. This, however, was not reflected in the root biomass, which did not differ between treatments indicating that the lower %N values were not a result of dilution by larger plants as might have been expected. At this time, however, the reason for this negative effect of N uptake is unknown.

Wheat exhibited the lowest inoculation success rate, with no shoots testing positive for *G. diazotrophicus* when the inoculant was applied as a DSA or RBA and no positive tests for the roots of plants receiving a foliar application. This suggests that the bacteria did not migrate beyond the application zone, which is the opposite of what Luna et al. (2010) reported for wheat and sorghum. In both studies the *G. diazotrophicus* was applied using a DSA method, though Luna et al. (2010) reported much higher colonization than that found in the current study, and which is likely why they also found greater *G. diazotrophicus* numbers in the above-ground plant tissues.

Three of the wheat replicates inoculated with Envita^{TM} as a foliar application were positive for *G. diazotrophicus,* indicating that the bacteria had colonized the shoot tissue, but the presence of the bacteria did not translate to BNF or increased %N in the shoots. Likewise, biomass was similarly unaffected by inoculation. These results presumably reflect the very low numbers of *G. diazotrophicus* detected in the shoots, which likely restricted BNF (Tian et al., 2009). On the other hand, wheat plants inoculated using the RBA method exhibited high *G. diazotrophicus* numbers in the roots but with only very small increases in ^{15}N concentration ($P \leq 0.05$). Likewise, the atom%

^{15}N in the shoots of plants receiving the RBA treatment was marginally greater ($P \leq 0.05$) than that in the control plants suggesting that small amounts of ^{15}N fixed in the roots was being translocated into the shoots. Much like the soybean, the percentage N in these wheat plants was lower than that in the controls while there were no differences in root biomass. Again, this suggests that the slight ^{15}N enrichment—and hence BNF—did not contribute to the N balance of the wheat plants.

Results for canola were the most inconsistent of the three plant species. All of the plants inoculated with the RBA method showed some very slight ^{15}N enrichment but not all tested positive for *G. diazotrophicus* colonization. Furthermore, though there was some evidence of the transfer of small amounts of ^{15}N by some means to the canola plants, again neither total plant N nor plant biomass were affected by inoculation with Envita™.

Data from the present study demonstrate that colonization of plant tissues (shoots or roots) by *G. diazotrophicus* does not by itself mean that the plants will derive an N benefit from the bacterium. For example, whereas 59% of the tissue samples examined tested positive for the presence of *G. diazotrophicus*, only about 7% of samples were significantly enriched in ^{15}N, with another 13% showing minor enrichments. It is interesting that soybean, the only legume tested, seemed to receive the most N benefit, though it may be that the physiology of the plant better enables it to receive and utilize N fixed by *G. diazotrophicus*. In a comparison of the effects of inoculation with *G. diazotrophicus*, Li and Macrae (1991) reported that corn provided a less favorable environment for *G. diazotrophicus* colonization than sugarcane due to its low-sugar content. And in a comparison of more than two dozen corn genotypes, Tian et al. (2009) reported a positive correlation between *G. diazotrophicus* colonization and the sucrose content of the plants. Whether this was a factor in the current study remains to be determined.

A slight ^{15}N enrichment in several of the plants that tested negative for *G. diazotrophicus* suggests that there may be some fixation and transfer of ^{15}N outside of the plant. Indeed, *G. diazotrophicus* is a free-living bacterium that can fix atmospheric N_2 without forming a plant association. Thus, very small amounts of fixed N were likely available to be taken up by the plant root systems. In general, however, the small magnitude of the enrichments suggests that this could simply be a function of the natural variability of the system.

Whereas my study failed to demonstrate a consistent N benefit of inoculation with *G. diazotrophicus* to soybean, wheat, or canola, it did provide insights into how plant colonization by the bacteria is affected by application method. Foliar applications of the *G. diazotrophicus*

generally had a high success rate of establishing colonization in the shoots of both soybean and canola but were considerably less effective for wheat. This was likely due to differences in shoot/leaf architecture, with soybean and canola providing a larger surface area for the foliar application. At the same time, successful colonization of the shoots did not necessarily translate into successful colonization of the roots of either soybean or canola.

In contrast, the DSA and RBA methods yielded successful inoculation of the roots, with the RBA method yielding more consistent results and with greater numbers of *G. diazotrophicus* being detected in the roots. As was the case with the foliar application, soybean responded well to the RBA of the inoculant; unlike with the foliar application, however, the shoots of plants inoculated with the RBA method also contained large numbers of *G. diazotrophicus*. This suggests that movement of the bacteria within the plant was largely unidirectional (i.e., from the roots to the shoots), occurring preferentially via the xylem. Neither the DSA nor RBA methods yielded successful inoculation of the canola. In the case of DSA, this likely reflects the small size (and hence small surface area) and hard coat of the canola seed limiting the number of bacteria on the seed and hindering access to the seed embryo. It was also suspected that the relatively low nutrient content of the canola seed may have hindered successful inoculation when *G. diazotrophicus* was applied using the RBA method.

For field applications, Azotic NA recommends that Envita^{TM} be applied directly to the seed, in-furrow, or as a foliar spray. In-furrow application works in a manner similar to the RBA method used in this study and data from this thesis suggest that in-furrow application may be the best choice for farmers growing soybean or wheat. For canola production, however, the data indicate that foliar application of the Envita™ inoculant is likely the best option. Ultimately, in terms of getting *G. diazotrophicus* into plant tissues, it might be possible to introduce the inoculant as a liquid formulation shortly after germination and emergence, though the probable timing restrictions and difficulty applying it are likely to be prohibitive. Certainly, more work is needed to (i) address inoculation strategies that would easily translate to the field-scale and (ii) how to ensure that successful colonization by the bacteria produces an N benefit to the crop.

6. CONCLUSION

Saskatchewan, as a global leader in crop production, has always been at the forefront of utilizing biological fertilizers such as the P-solubilizing fungus *Penicillium bilaiae* (Asea et al., 1988; Knight, 2020). Today there is an emerging market of bio-based fertilizers that rely on asymbiotic N_2 fixation to provide a significant N benefit to a variety of crops (Franzen et al., 2023). One such product, Envita™, employs the naturally-occurring bacterium *G. diazotrophicus* to deliver biologically fixed N to crops such as corn, soybean, and wheat (Cooking et al., 2006). And though it has been amply demonstrated that *G. diazotrophicus* is capable of fixing atmospheric N_2 (Cavalcante & Dobereiner, 1988; Eskin et al., 2013; Chawla et al. 2014) the N and yield benefits derived from its use have been inconsistent at best. Nevertheless, BNF products like Envita™ will undoubtedly play a crucial role in shaping the practices of future agriculture as relying solely on a single type of fertilizer is not sustainable in an ever-evolving future. Therefore, having diverse range of fertilizer options, whether synthetic or biological, will be vital to fostering sustainability within the agriculture industry.

In the present study it was demonstrated that Envita™ could deliver the free-living, N-fixing bacterium *G. diazotrophicus* to roots and shoots of wheat, canola, and soybean; and that the bacteria were able to colonize the tissues in many, but not all cases. However, the mere presence of the *G. diazotrophicus* in the shoot and root tissues often did not translate into $^{15}N_2$ being fixed into the plant tissues. Foliar application of the inoculant in soybean (three of five plants) grown in the semi-sterile Leonard jars provided the strongest evidence of the inoculant inducing biological N fixation. But when inoculation was performed in non-sterile soil systems where other soil-borne microorganisms presumably compete with the introduced *G. diazotrophicus*, evidence of BNF was generally lacking. To further complicate matters, when tissues were slightly enriched in ^{15}N indicating probable BNF, the %N in the tissues tended to be unchanged or reduced compared to the control, with no change in biomass production. In these cases, it seems that the *G. diazotrophicus* itself may be preferentially using N the fixed N at the expense of the plants.

Of the three inoculation methods evaluated soaking the seed in inoculant during germination was the most effective at introducing the *G. diazotrophicus* for tissue colonization, followed by foliar application and finally applying the inoculant to the seed. Unfortunately, the germination soaking method is also the least practical to implement on a large scale, and though it may be transferable as a soil drench or in-furrow application of liquid inoculant, it is difficult to imagine that this could be implemented on a wide scale given the time restrictions that farmers are faced with during seeding and spraying.

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APPENDIX A

VERIFYING THE COLONIZATION OF PLANT TISSUES BY GLUCONACETOBACTER DIAZOTROPHICUS

A.1 Objective

A team from the National Research Council (NRC) developed a new method to verify the colonization of *Gluconacetobacter diazotrophicus* in plant tissues to confirm successful inoculation of the target crops with the commercial *G. diazotrophicus* inoculant Envita™. Plant tissues from this thesis research were provided to the NRC team to verify its utility as a method for quantifying bacterial colonization.

A.2 Materials and Methods

A.2.1 Sample collection and preparation

Plants were grown in a controlled environment chamber and at harvest the shoot tissues were subsampled and placed into 50-mL Falcon tubes (Fisher Scientific, Mississauga, ON). Next, any soil on the roots was gently removed from each individual plant sample and transferred into a 500-mL Erlenmeyer flask containing 200 mL of sterile 1× phosphate-buffered saline (PBS) buffer. The flasks were then placed on a rotary shaker at 150 rpm and shaken for 25 min at 22°C. The plant roots were rinsed with distilled water until clean and then with 800 mL of sterilized distilled water. Each cleaned root was dried with a clean Kimwipe and placed into a new 50-mL Falcon tube. Finally, the plant samples were freeze-dried using the Labconco™ Freezone 6-Liter Freeze-Dry System (NRC, 2021).

A.2.2 DNA extraction

Eight 6.3 mm steel balls were placed into the freeze-dried sample tube which was then placed into the SPEX SamplePrep 2010 Geno/Grinder at 1200 rpm for 4 to 8 minutes. Ten to fifteen milligrams of the ground material was used to extract genomic DNA using the DNeasy Plant Pro

Kit (Qiagen) following the manufacturer's instructions. Subsequently, the concentration of the extracted DNA was determined using the Qubit dsDNA HS Assay (Thermo Fisher Scientific).

A.2.3 Droplet digital PCR assay (ddPCR)

EvaGreen assay (Bio-Rad) Droplet digital PCR was performed using the EvaGreen assay (Bio-Rad) on a Bio-Rad QX200 system. The ddPCR reaction mixture (25 µL) contained 1X EvaGreen Supermix, 100 nM of forward and reverse primers (table x), and 2 to 10 µL of DNA template. Afterwards, the 20 µL mixed and centrifuged PCR reactions were transferred into the sample well of a DG8 cartridge. Next, droplets were generated using the QX200 generator by adding 70 µL of QX200 droplet generation oil into the oil wells and covering the cartridge with a DG8 gasket. The droplets were then transferred into a PCR plate using a multi-channel pipette, and the PCR plates were sealed using PCR plate heat seal foil and a PX1 PCR plate sealer. PCR was performed on a C100 touch thermal cycler (Bio-Rad) using a 3-step PCR protocol: 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 60°C to 62°C for 30 seconds, and 72°C for 30 seconds; followed by 4°C for 5 minutes and 90°C for 5 minutes; with a ramp rate set to 2.5°C per second. Finally, the treated PCR plates were later counted in a QX200 droplet reader for positive and negative droplets. Additionally, thresholds to separate positive from negative droplets were set manually and the data were analyzed using QuantaSoft software (Bio-Rad).

Table A.1. Primers used for ddPCR.

Primer Name	Sequence (5' → 3')	Target
H2190	ATG GCC AGC AAA GAC GTC AA	Forward primer, targeting *Gd* cpn60.2
H2191	TGT CGA TTC CGG AAA GCA GG	Reverse primer, targeting *Gd* cpn60.2

A.3 Results

The results of the ddPCR analyses are presented in Table 8.2, Table 8.3, and Table 8.4. The interpretation of *Gd* detection was determined based on three levels: **negative** *Gd* detection (0 to 4 *Gd* copies 100-ng^{-1} DNA), **borderline** *Gd* detection (4–5 *Gd copies* 100-ng^{-1} DNA), and **positive** *Gd* detection (>5 *Gd* copies 100-ng^{-1} DNA).

Table A.2. Comprehensive results of Control soybean sample, Foliar applied Envita™ soybean samples, Direct seed applied Envita™ soybean samples, and Root bath applied Envita™ soybean samples. Samples were pulse labelled with $^{15}N_2$ in a closed chamber for 24hours and analysed by NRC for colonization of *G.d* and $^{15}N_2$ analysis using mass spec technique.

Treatment	Gd 100-ng^{-1} gDNA‡	Gd status†	N%	Atom%^{15}N
		Shoots		
S1 Control	4	±	1.32	0.3699
S2 Control	1	-	1.47	0.3697
S3 Control	2	-	1.61	0.3694
S4 Control	5	±	1.20	0.3700
S5 Control	5	±	1.39	0.3699
S1 Foliar	2800	+	1.57	0.3697
S2 Foliar	2875	+	1.39	0.3702
S3 Foliar	3575	+	1.69	0.3694
S4 Foliar	3450	+	1.70	0.3699
S5 Foliar	4300	+	1.71	0.4076
S1 DSA	8	+	1.35	0.3701
S2 DSA	2	-	1.65	0.3696
S3 DSA	1	-	1.33	0.3693
S4 DSA	2	-	1.71	0.4257
S5 DSA	4	±	1.42	0.3695
S1 RBA	56	+	1.14	0.3706
S2 RBA	49	+	1.17	0.3700
S3 RBA	1709	+	1.76	0.3702
S4 RBA	189	+	1.16	0.3706
S5 RBA	165	+	1.50	0.4772
		Roots		
S1 Control	4	±	1.75	0.3701
S2 Control	1	-	1.82	0.3699
S3 Control	2	-	2.21	0.3696
S4 Control	3	-	1.46	0.3697
S5 Control	3	-	1.99	0.3695
S1 Foliar	13	+	1.70	0.3694
S2 Foliar	8	+	1.90	0.3701
S3 Foliar	49	+	2.23	0.3694
S4 Foliar	6	+	2.01	0.3697
S5 Foliar	24	+	1.87	0.3841
S1 DSA	59	+	1.67	0.3700
S2 DSA	16	+	1.88	0.3695
S3 DSA	71	+	1.68	0.3690
S4 DSA	1	-	1.80	0.3945
S5 DSA	89	+	1.83	0.3695
S1 RBA	731	+	1.29	0.3705
S2 RBA	216	+	1.43	0.3696
S3 RBA	70	+	1.52	0.3701
S4 RBA	1579	+	1.19	0.3702
S5 RBA	992	+	1.66	0.4373

† Positive response (+); broadline response (±); negative response (-).

‡ gDNA is short for Genomic DNA, which is the total genetic information of an organism.

Table A.3. Comprehensive results of Control wheat sample, Foliar applied Envita™ wheat samples, Direct seed applied Envita™ wheat samples, and Root bath applied Envita™ wheat samples. Samples were pulse labelled with $^{15}N_2$ in a closed chamber for 24hours and analysed by NRC for colonization of *G.d* and $^{15}N_2$ analysis using mass spec technique.

Treatment	Gd 100-ng^{-1} gDNA	Gd status	N%	Atom%^{15}N
		Shoots		
W1 Control	1	-	1.71	0.3712
W2 Control	0	-	1.72	0.3712
W3 Control	0	-	1.79	0.3710
W4 Control	0	-	1.71	0.3712
W5 Control	0	-	1.84	0.3712
W1 Foliar	10	+	1.59	0.3711
W2 Foliar	10	+	1.67	0.3713
W3 Foliar	3	-	1.60	0.3715
W4 Foliar	2	-	1.56	0.3712
W5 Foliar	9	+	1.60	0.3711
W1 DSA	2	-	1.78	0.3714
W2 DSA	0	-	1.74	0.3712
W3 DSA	2	-	1.86	0.3717
W4 DSA	2	-	1.74	0.3713
W5 DSA	0	-	1.61	0.3711
W1 RBA	1	-	1.68	0.3722
W2 RBA	0	-	1.74	0.3716
W3 RBA	2	-	1.68	0.3717
W4 RBA	0	-	1.70	0.3718
W5 RBA	0	-	1.59	0.3715
		Roots		
W1 Control	0	-	1.46	0.3708
W2 Control	0	-	1.04	0.3707
W3 Control	0	-	1.21	0.3706
W4 Control	0	-	1.15	0.3707
W5 Control	0	-	1.44	0.3708
W1 Foliar	0	-	1.22	0.3707
W2 Foliar	2	-	1.22	0.3709
W3 Foliar	4	±	1.05	0.3709
W4 Foliar	5	±	1.06	0.3708
W5 Foliar	0	-	1.22	0.3706
W1 DSA	181	+	1.28	0.3709
W2 DSA	11	+	1.21	0.3706
W3 DSA	39	+	1.28	0.3711
W4 DSA	25	+	1.26	0.3708
W5 DSA	4090	+	1.22	0.3707
W1 RBA	136	+	1.42	0.3714
W2 RBA	816	+	1.30	0.3710
W3 RBA	316	+	1.30	0.3711
W4 RBA	2110	+	1.28	0.3712
W5 RBA	4362	+	1.21	0.3709

† Positive response (+); broadline response (±); negative response (-).

‡ gDNA is short for Genomic DNA, which is the total genetic information of an organism.

Table A.4. Comprehensive results of Control canola sample, Foliar applied Envita™ canola samples, Direct seed applied Envita™ canola samples, and Root bath applied Envita™ canola samples. Samples were pulse labelled with $^{15}N_2$ in a closed chamber for 24hours and analysed by NRC for colonization of *G.d* and $^{15}N_2$ analysis using mass spec technique.

Treatment	Gd 100-ng^{-1} gDNA	Gd status[a]	N%	Atom%^{15}N
		Shoots		
C1 Control	11	+	1.00	0.3725
C2 Control	10	+	0.92	0.3728
C3 Control	3	-	1.13	0.3724
C4 Control	7	+	1.08	0.3722
C5 Control	7	+	1.10	0.3722
C1 Foliar	3370	+	0.95	0.3721
C2 Foliar	5130	+	1.09	0.3725
C3 Foliar	5350	+	1.15	0.3723
C4 Foliar	4390	+	1.02	0.3722
C5 Foliar	4620	+	1.15	0.3721
C1 DSA	20	+	1.01	0.3722
C2 DSA	8	+	0.98	0.3724
C3 DSA	5	±	0.87	0.3721
C4 DSA	9	+	0.96	0.3722
C5 DSA	7	+	1.14	0.3724
C1 RBA	26	+	0.92	0.3733
C2 RBA	3	-	1.26	0.3731
C3 RBA	5	±	1.16	0.3729
C4 RBA	1	-	1.00	0.3733
C5 RBA	9	+	1.04	0.3732
		Roots		
C1 Control	0	-	1.83	0.3710
C2 Control	0	-	1.66	0.3711
C3 Control	0	-	1.65	0.3709
C4 Control	0	-	1.71	0.3709
C5 Control	0	-	1.68	0.3710
C1 Foliar	6	+	1.79	0.3710
C2 Foliar	5	±	1.80	0.3711
C3 Foliar	10	+	1.74	0.3710
C4 Foliar	0	-	1.81	0.3709
C5 Foliar	6	+	2.06	0.3710
C1 DSA	0	-	1.71	0.3708
C2 DSA	0	-	1.73	0.3709
C3 DSA	0	-	1.81	0.3708
C4 DSA	4	±	1.72	0.3709
C5 DSA	5	±	1.76	0.3711
C1 RBA	8	+	1.72	0.3715
C2 RBA	5	±	1.58	0.3715
C3 RBA	3	-	1.45	0.3715
C4 RBA	2	-	1.55	0.3717
C5 RBA	3	-	1.69	0.3718

[†] Positive response (+); broadline response (±); negative response (-).

[‡] gDNA is short for Genomic DNA, which is the total genetic information of an organism.